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(54) Title: HUMAN PROGASTRIN FOR THE DIAGNOSIS AND TREATMENT OF PATHOLOGICAL CONDITIONS OF THE GASTROINTESTINAL TRACT

(57) Abstract

This invention relates to methods and compositions utilizing progastrin for the diagnosis and treatment of pathological conditions of the gastrointestinal tract, and in particular of conditions associated with proliferative or degenerative diseases of the gastrointestinal mucosa. In one embodiment the invention provides methods and compositions utilizing recombinant human progastrin$_{6-80}$ for diagnosis and treatment of conditions associated with hyperprogastrinaemia, including colorectal carcinoma, duodenal ulcer, and gastrinoma, and side effects of treatment of gastric ulcer with ATPase blockers. In an alternative embodiment, the invention provides methods and compositions utilizing recombinant human progastrin$_{6-80}$ for treatment of degenerative diseases of the gastrointestinal mucosa, such as ulcerative colitis and inflammatory bowel disease.

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HUMAN PROGASTRIN FOR THE DIAGNOSIS AND TREATMENT OF PATHOLOGICAL CONDITIONS OF THE GASTROINTESTINAL TRACT

This invention relates to methods and compositions for the diagnosis and treatment of pathological conditions of the gastrointestinal tract, and in particular of conditions associated with proliferative or degenerative diseases of the gastrointestinal mucosa. In one embodiment the invention provides methods and compositions for diagnosis and treatment of conditions associated with hyperprogastrinaemia, including colorectal carcinoma, duodenal ulcer, and gastrinoma, and side-effects of treatment of gastric ulcer with ATPase blockers. In an alternative embodiment, the invention provides methods and compositions for treatment of degenerative diseases of the gastrointestinal mucosa, such as ulcerative colitis and inflammatory bowel disease.

BACKGROUND OF THE INTRODUCTION

Gastrin is a classical gut peptide hormone which was originally identified as a stimulant of gastric acid secretion. Like many other peptide hormones, gastrin is initially synthesized as a large precursor molecule of 101 amino acids, whose sequence is shown in Figure 1; this precursor is rapidly converted to progastrin (80 amino acids) by cleavage of the N-terminal signal peptide. Progastrin is further extensively processed by endo-and carboxypeptidases to yield the final end products glycine-extended $gastrin_{17}$ and $gastrin_{17}$ (Dockray *et al*, 1996).

The major progastrin-derived peptides in human antrum are $progastrin_{1-35}$, $progastrin_{6-35}$ and $progastrin_{20-35}$ (Rehfeld and Johnsen, 1994). The sequence of these peptides is consistent with the cleavage of progastrin, firstly by an enzyme recognising monobasic Arg (R) sites at positions 5 and 19, and secondly by an enzyme recognising dibasic ArgArg (RR) sites at positions 36-37 and 73-74 (Figure 1). Cleavage by the first enzyme only would be expected to produce $progastrin_{6-80}$ and $progastrin_{20-80}$ from $progastrin_{1-80}$.

Hence progastrin$_{6-80}$ is expected to occur *in vivo*.

Although gastrin$_{17}$ was originally thought to be the only form of the hormone with biological activity, glycine-extended gastrin$_{17}$ has recently been shown to stimulate the proliferation of several cell lines (Seva *et al*, 1994; Singh *et al*, 1996; Hollande *et al*, 1997). Furthermore progastrin itself appears to act as a growth factor for normal colon, since transgenic mice expressing progastrin in the liver have elevated levels of serum progastrin and a hyperplastic colonic mucosa (Wang *et al*, 1996).

At least four classes of receptors for the related hormones gastrin and cholecystokinin (CCK) have been described. The CCK-A receptor on the pancreatic acinar cell, and the gastrin/CCK-B receptor on the gastric parietal cell, both belong to the family of receptors with seven transmembrane segments (Silvente-Poirot *et al*, 1993). Peptide binding to either the CCK-A receptor (Gardner *et al*, 1985), or to the gastrin/CCK-B receptor (Magous *et al*, 1982; Matsumoto *et al*, 1987), requires an amidated C-terminal residue. A low-affinity gastrin binding site has also been described on the surface of gastric carcinoma (Weinstock *et al*, 1988) and colonic carcinoma (Hoosein *et al*, 1988) cell lines, and called the gastrin/CCK-C receptor (Baldwin, 1994). The gastrin/CCK-C receptor is related in sequence to a family of proteins involved in fatty acid oxidation (Baldwin, 1993), and binds amidated and C-terminally extended forms of gastrin with equal affinity (Baldwin, 1995). Two novel receptors for glycine-extended gastrin have recently been described on the rat pancreatic carcinoma cell line AR4-2J (Seva *et al*, 1994), on Swiss 3T3 fibroblasts (Singh *et al*, 1995) and on the mouse colon cell line YAMC (Hollande *et al*, 1997).

The possibility that progastrin-derived peptides might stimulate the proliferation of colon carcinoma cells via an autocrine loop has recently received considerable attention. The autocrine model predicts that a cell

synthesizes a particular growth factor, which, after release into the surrounding medium, binds to specific receptors on the surface of the same cell, and stimulates the proliferation of that cell. The observation that expression of antisense gastrin mRNA inhibits proliferation of a non-transformed colon cell line *in vitro* (Hollande, *et al*, 1997) and of tumorigenic colorectal carcinoma cell lines *in vitro* and *in vivo* (Singh *et al*, 1996) provides strong evidence that progastrin-derived peptides may act as autocrine growth factors in colorectal carcinoma.

As predicted by the autocrine model, most colon carcinomas and derived cell lines synthesize gastrin mRNA and progastrin-derived peptides. The amounts of gastrin mRNA are low, and can only be detected by PCR (Baldwin and Zhang, 1992; Van Solinge *et al*, 1993) or by RNase protection (Imdahl *et al*, 1995). However, progastrin-derived peptides have been detected directly in colorectal carcinomas by immunohistochemistry (Finley *et al*, 1993), and in extracts from colon carcinomas and colon carcinoma cell lines by radioimmunoassay (Van Solinge *et al*, 1993; Kochman *et al*, 1992; Nemeth *et al*, 1993). One or more processing enzymes appears to be absent from colorectal carcinomas, since mature amidated gastrin is generally not present (Van Solinge *et al*, 1993; Kochman *et al*, 1992; Nemeth *et al*, 1993). Although all of a panel of five colon carcinoma cell lines synthesized progastrin-derived peptides, only two released the peptides into the medium (Van Solinge *et al*, 1993). The elevated levels of progastrin-derived peptides detected in the sera of patients with colorectal carcinoma may therefore arise from a source other than the tumour (Ciccotosto *et al*, 1995).

The identity of gastrin receptors on colorectal carcinomas is still unclear. Despite considerable controversy, the current consensus is that only 10% of colorectal carcinomas and derived cell lines express gastrin/CCK-B receptors. Although an early report detected high-affinity $gastrin_{17}$ binding sites characteristic of the

gastrin/CCK-B receptor on 57% of colorectal carcinomas (Upp *et al*, 1989), a recent study detected no high affinity gastrin$_{17}$ binding in 112 resected tumours (Imdahl *et al*, 1995). Furthermore mRNA encoding the gastrin/CCK-B receptor was detected in only 11% of tumour samples by RNase protection assay (Imdahl *et al*, 1995) and in 20% of samples by Northern blotting (Matsushima *et al*, 1994). High-affinity binding sites for gastrin$_{17}$ were detected on only 10% of human colorectal carcinoma cell lines (Frucht *et al*, 1992), and mRNA encoding the gastrin/CCK-B receptor has been detected in only 1 of 8 colorectal carcinoma cell lines tested (Matsushima *et al*, 1994). Although gastrin/CCK-C receptors were detected on all colorectal carcinoma cell lines tested (Weinstock *et al*, 1988; Hoosein *et al*, 1988), low-affinity binding characteristic of the gastrin/CCK-C receptor was observed in only 30% of tumour specimens (Imdahl *et al*, 1995). Since most colorectal carcinomas do not express either gastrin/CCK-B and -C receptors, there is an urgent need to determine whether other receptors for progastrin-derived peptides are present on tumour specimens.

No information is yet available on the binding of progastrin itself to any gastrin/CCK receptor. We have therefore developed a method for expression of progastrin$_{6-80}$ in *E. coli*, in order to purify the large amounts of progastrin required for direct testing of its affinity for known gastrin/CCK receptors, its mitogenic effects on cell lines, and hence its possible involvement in an autocrine loop. We have surprisingly found that progastrin binds to gastrin/CCK-C receptors, but not to gastrin/CCK-A receptors or to gastrin/CCK-B receptors, and that binding stimulates proliferation of a non-transformed colon cell line and of colorectal carcinoma cell lines in a dose-dependent manner.

SUMMARY OF THE INVENTION

Our results indicate that progastrin is involved in an autocrine mechanism for the control of proliferation and replacement of cells of the gastrointestinal mucosa, especially the colonic mucosa, and is involved in proliferation of cancers of the gastrointestinal mucosa.

In a first aspect, the invention provides a purified recombinant progastrin molecule. Preferably the recombinant progastrin comprises amino acid residues 6 to 80 of the complete progastrin sequence.

It will be clearly understood that recombinant human progastrin$_{1-80}$, or fragments thereof capable of binding to the same receptor as progastrin$_{6-80}$, are also within the scope of the invention. Such fragments are referred to herein as "biologically-active fragments".

In a preferred embodiment, the recombinant progastrin is expressed as a fusion protein with glutathione-S-transferase, purified by affinity chromatography on glutathione-agarose beads, and cleaved from the fusion protein by incubation with thrombin. More preferably the recombinant progastrin is purified by reverse phase high performance liquid chromatography following the cleavage with thrombin. Any suitable expression host may be used, including but not limited to bacterial cells, such as *Escherichia coli*, yeast cells such as *Saccharomyces cerevisiae* or *Pichia pastoris*, insect cells, or mammalian cells. The person skilled in the art will be able to choose a suitable host cell.

In a second aspect, the invention provides an antibody directed against progastrin. Preferably the antibody binds to a region of the progastrin molecule within the sequence comprising amino acid residues 6 to 80. More preferably the antibody inhibits binding of progastrin to cell-surface receptors so as to inhibit progastrin-induced cell proliferation. The person skilled in the art will be aware of standard methods for production of both polyclonal and monoclonal antibodies, and antigen-binding

derivatives thereof such as $(Fab)_2$ fragments. The person skilled in the art will also be aware that "humanized" monoclonal antibodies and biologically-active antibody derivatives such as ScFv fragments and divalent ScFv-type molecules can be prepared using recombinant methods. The antibody may be labelled with a detectable marker, which is suitably a radioactive label, such as radioactive iodine, or may be a fluorescent or chemiluminescent label. A person skilled in the art will be able to select suitable radioactive, fluorescent or chemiluminescent labels.

It will be clearly understood that compositions comprising the recombinant progastrin and the antibody of the invention are also within the scope of the invention. Such compositions comprise the progastrin or the antibody together with a pharmaceutically-acceptable carrier. A person skilled in the art will be aware of suitable carriers.

Suitable formulations for administration by any desired route may be prepared by standard methods, for example by reference to well-known text such as Remington; The Science and Practice of Pharmacy, Vol. II, 1995 (19^{th} edition), A.R. Gennaro (ed), Mack Publishing Company, Eastern Pennsylvania, or Australian Prescription Products Guide, Vol. 1, 1995 (24^{th} edition) J. Thomas (ed), Australian Pharmaceutical Publishing Company Ltd, Victoria, Australia.

According to a third aspect the invention provides a method of diagnosis of a condition associated with hyperprogastrinaemia, comprising the step of detecting progastrin in a biological sample.

In an alternative aspect the invention provides a method of diagnosis of a condition associated with hyperactivity of autocrine stimulation, proliferation or activity of cells of the gastrointestinal mucosa, comprising the step of detection of elevated levels of progastrin in a biological sample.

Preferably the condition is a cancer of the gastrointestinal tract, such as colorectal carcinoma, or a condition associated with hyperprogastrinaemia, such as duodenal ulcer or gastrinoma.

Preferably progastrin is detected using an immunoassay; a variety of immunoassay methods may be used, including but not limited to radioimmunoassay or enzyme-linked immunosorbent assay. The immunoassay may utilise a polyclonal antibody or monoclonal antibody or an antigen-binding fragment thereof, but a monoclonal antibody is preferred. In either case, the antibody preferably binds to a region of the progastrin molecule within the sequence comprising amino acid residues 6 to 80.

The biological sample may be blood, plasma, or serum, or may be a tissue sample. Where a tissue sample is used, progastrin may be detected using an immunoassay performed on a cell or tissue extract, or may utilise immunohistochemical techniques employing a polyclonal or monoclonal antibody labelled with a detectable marker. The detectable marker is suitably a radioactive label, such as radioactive iodine, or may be a fluorescent or chemiluminescent label. A person skilled in the art will be able to select suitable radioactive, fluorescent or chemiluminescent labels. Immunohistochemical methods of the invention preferably utilise monoclonal antibodies as described above.

The invention also contemplates a diagnostic kit comprising an antibody to progastrin, labelled with a detectable marker as described above.

In a fourth aspect, the invention provides a method of treatment of a condition associated with hyperactivity of autocrine stimulation of proliferation of cells of the gastrointestinal mucosa, comprising the step of administering an effective amount of an antagonist of binding of progastrin to gastrin/CCK-C receptors or other progastrin receptors to a mammal in need of such treatment.

Preferably the condition is associated with hyperprogastrinaemia. These conditions include, but are not limited to, colorectal carcinoma, duodenal ulcer, gastrinoma, and side-effects of treatment with ATPase blockers (proton pump inhibitors) such as substituted benzimidazoles, including but not limited to omeprazole and pantoprazole.

Suitable antagonists include antibodies directed against progastrin, preferably monoclonal antibodies and biologically-active fragments or recombinant derivatives thereof.

In a fifth aspect, the invention provides a method of treatment of a degenerative condition of the gastrointestinal mucosa, comprising the step of administering an effective amount of recombinant human progastrin to a mammal in need of such treatment, in order to stimulate restorative proliferation of the cells of the damaged mucosa. The degenerative condition may include, but is not limited to, ulcerative colitis and inflammatory bowel disease.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

Brief Description of the Figures

Figure 1 shows the structure of the glutathione S-transferase (GST)-progastrin fusion protein compared with the structures of naturally-occurring progastrin-derived peptides. Amino acids are shown in the one letter code, with progastrin sequences in upper case and linker sequences in lower case. Numbering commences at the N-terminus of mature progastrin. Thrombin cleavage sites are indicated by vertical arrows.

Figure 2 shows the results of SDS-electrophoresis of a progastrin fusion protein. Human progastrin was expressed in *Escherichia coli* as a fusion protein with

glutathione S-transferase. The fusion protein was purified from bacterial lysates by chromatography on glutathione-agarose, and cleaved by treatment with thrombin. Samples from the indicated stages of the purification were electrophoresed on 10% SDS polyacrylamide gels and visualised by staining with Coomassie blue. 1, bacterial lysate; 2, glutathione-agarose run through; 3, glutathione-agarose bound material; 4, glutathione-agarose bound material after thrombin cleavage; 5, glutathione-agarose supernatant after thrombin cleavage; 6, glutathione-S-transferase; 7, molecular weight markers (size in kDa; O=origin; DF=dye front).

Figure 3 shows the results of human progastrin purified from thrombin digests of the glutathione-S-transferase-progastrin fusion protein by reverse phase HPLC. Protein was detected by absorbance at 214 nm, and progastrin by radioimmunoassay with antibody 1137, which recognises the C-terminal decapeptide.

Figure 4 shows the results of binding studies on recombinant human progastrin. Binding of recombinant human progastrin$_{6-80}$ (closed squares) to COS cells expressing the human CCK-A (A) and gastrin/CCK-B (B) receptors was measured by competition with [^{125}I]-CCK$_8$ (30 pM, 20000 cpm). Values were expressed as a percentage of the value obtained in the absence of competitor. Binding of CCK$_8$ (A, open squares) and gastrin$_{17}$ (B, open circles) was measured as a control. Points are the mean ± SEM of triplicates from 3 experiments.

Figure 5 illustrates binding of recombinant human progastrin to the gastrin/CCK-C receptor. Binding of recombinant human progastrin$_{6-80}$ (closed squares) to the porcine gastrin/CCK-C receptor was measured by competition with [^{125}I]-Nle^{15}-gastrin$_{2,17}$ (30 pM, 20000 cpm) in a covalent cross-linking assay. Values were expressed as a percentage of the value obtained in the absence of competitor.

Figure 6 shows the effects of progastrin (PG) and gastrin$_{17}$gly (Ggly) on proliferation of YAMC mouse colon

cells. Proliferation was measured by the MTT assay for 5 days after seeding (open circles). The mean of quadruplicate absorbance readings was calculated for each sample. Results are expressed as a percentage of the mean absorbance readings obtained for untreated cells; error bars represent the standard error of the mean from three separate experiments.

Figure 7 shows the effects of progastrin (PG) and $gastrin_{17}gly$ (Ggly) on proliferation of human gastrointestinal carcinoma cell lines.

a. LIM1215 colorectal carcinoma cells
b. LIM1899 colorectal carcinoma cells.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of reference only to the following non-limiting examples, and to the drawings.

It will be clearly understood that while the invention is described in detail in relation to $progastrin_{6-80}$, the invention is not limited to this form of progastrin.

In particular since the data presented herein demonstrate that $progastrin_{6-80}$ is biologically active, and since the entire sequence of $progastrin_{6-80}$ is contained within the sequence of $progastrin_{1-80}$, it is reasonable to assume that $progastrin_{1-80}$ will also be biologically active. Moreover antibodies against $progastrin_{6-80}$ will be equally efficacious in measurement of $progastrin_{1-80}$, and antagonists which interefere with the binding of $progastrin_{6-80}$ to its receptor will also interfere with the binding of $progastrin_{1-80}$ to the same receptor.

Abbreviations used herein are as follows:

DTT	dithiothreitol
CCK	cholecystokinin
FCS	foetal calf serum

$Gastrin_{17}gly$	glycine-extended $gastrin_{17}$
GST	glutathione S-transferase;
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
HPLC	high performance liquid chromatography
IC_{50}	concentration required for 50% inhibition
IPTG	isopropylthiogalactoside;
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
PBS	phosphate-buffered saline
SDS	sodium dodecyl sulphate

Statistics

Results are expressed as mean ± SE, except where otherwise stated. Comparisons between groups were made by Student's t test. Differences with P values of < 0.05 were considered significant.

Example 1 Expression of human progastrin in *Escherichia coli*

Human progastrin was expressed in *E. coli* as a fusion protein with glutathione-S-transferase.

A Hind III-Hind III fragment of human gastrin cDNA, corresponding to nucleotides 59-325 of the sequence reported by Boel and coworkers (Boel *et al*, 1983), and hence encoding the entire sequence of mature human progastrin 1-80 (Rehfeld *et al*, 1994), was subcloned into Hind III-cleaved and dephosphorylated pGEX-2TH (Smith and Johnson, 1988). Clones with the insert in the correct orientation were selected by restriction mapping. The predicted sequence of the fusion protein, which was confirmed by nucleotide sequencing, consisted of glutathione S-transferase (GST) joined to progastrin 1-80 by a 6 amino acid linker of sequence GSEFQA arising from the multiple cloning site.

The GST-progastrin fusion protein was purified from Sarkosyl lysates of *E. coli* by binding to glutathione-

agarose as described by Frangioni and Neel (1993). Briefly, *E. coli* strain NM522 was transformed with the plasmid of interest and grown overnight at 37°C with shaking in LB medium containing 100 µg/ml of ampicillin. The overnight culture (40 ml) was used to inoculate the same medium (360 ml). When an absorbance at 600 nm of 0.8 was reached the expression of progastrin was induced by treatment with 0.1 mM IPTG for 6 hrs. The cells were harvested by centrifugation at 2500 g for 10 min. The cell pellet was washed in cold STE buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA), and resuspended in 24 ml STE containing 100 µg/ml lysozyme. After incubation on ice for 15 min. DTT was added to 5 mM and proteins were solubilised with 1.5% Sarkosyl (Sigma, St. Louis, MO). After vortexing for 15 sec, cells were sonicated for 2 x 30 sec (power level 4, duty cycle 50%) in a Model 250 Sonifier (Branson Sonic Power Co., Danbury, CT). The lysate was clarified by centrifugation at 2500 g for 5 min at 4°C. The supernatant was transferred to a new tube and Triton X-100 was added to 2%. After vortexing for 10 sec washed glutathione-agarose beads (200 µl, 50% v/v suspension in PBS) were added and the suspension was gently mixed by rotation at 4°C for 1 hour. The beads were then washed 3 times with ice-cold PBS by repeated low speed centrifugation and resuspension in PBS. Finally the beads with GST-fusion proteins attached were stored at -70°C in storage buffer (50 mM Na^+ Hepes, pH 7.4, 150 mM NaCl, 5 mM DTT, 10% v/v glycerol).

Recombinant human progastrin was cleaved from the GST-progastrin fusion protein bound to glutathione-agarose beads by incubation with thrombin (Sigma) in 100 µl cleavage buffer (50 mM Hepes, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$) for 1 hour at 37°C. Following cleavage, the supernatant containing progastrin was separated from the beads by centrifugation. The beads were washed twice with 200 µl elution buffer (1 M urea, 50 mM Hepes, pH 7.5), and the washes were combined with the initial supernatant.

Figure 2 shows the results of SDS-polyacrylamide gel electrophoresis of samples from the different stages of purification.

In particular the GST-progastrin fusion protein isolated by chromatography on glutathione-agarose is shown in track 3, and the progastrin$_{6-80}$ generated by cleavage of the fusion protein with thrombin is shown in track 5.

Interestingly, the yields of fusion protein as determined by radioimmunoassay with an antiserum specific for the C-terminal hexapeptide of progastrin were much lower than the yields estimated by Coomassie blue staining.

Example 2 Purification and Characterization of Recombinant Human Progastrin

Recombinant human progastrin was purified by reverse phase-HPLC. The recombinant human progastrin prepared in Example 1 was diluted in 1.0 ml 0.05 M ammonium bicarbonate/20% acetonitrile and applied to a C18 column (8 x 100 mm, Waters Associates, Milford MA), which had been equilibrated with 0.05 M ammonium bicarbonate/20% acetonitrile. The progastrin was eluted with a gradient of from 20-50% acetonitrile in 0.05 M ammonium bicarbonate at a flow rate of 1 ml·min^{-1}. Fractions of 0.5 ml were collected and dried on a Speedvac for radioimmunoassay, mass spectrometry and peptide sequencing.

The concentrations of recombinant human progastrin in chromatographic fractions were measured by radioimmunoassay, using a polyclonal antiserum designated 1137, raised in rabbits against an undecapeptide consisting of the C-terminal gastrin decapeptide (residues 71-80) with an additional tyrosine residue at the N-terminus for iodination as previously described (Ciccotosto *et al*, 1995). A C-terminal flanking peptide standard curve was constructed with ^{125}I-C-terminal flanking peptide as label. The ID_{50} was 1.3 ± 0.2 fmol/tube, and the intraassay variation was < 7%.

As shown in Figure 3, the absorbance peak at fraction 21-22 matched very well with the peak of immunoactivity observed with antiserum 1137. The conclusion that the recombinant human progastrin contained the C-terminus of progastrin was confirmed by mass spectrometry.

Samples (2ml-4ml) from reverse phase HPLC fractions were analysed directly by electrospray ionisation mass spectrometry using a Perkin-Elmer Sciex API-300 triple quadrupole mass spectrometer fitted with a micro-ionspray ion source. Samples were infused through 20 µm I.D. fused silica tubing using a 10 µl Hamilton gas-tight syringe driven by a Harvard syringe pump at a flow rate of 0.2 µl/min. Signal-averaged spectra were obtained from 50-100 scans over 5-10 mins, using a scan range of m/z100-m/z2500 and a constant peak width (at half height) of 0.6 amu. Prior to sample analysis, peak widths were adjusted and the mass scale calibrated to an accuracy of 0.01% using singly-charged poly(propyleneglycol) reference ions.

The molecular mass of HPLC-purified recombinant human progastrin was 8427.1 Da, which is in exact agreement with the mass predicted for human progastrin$_{6-80}$.

N-terminal amino acid sequences were obtained by sequential Edman degradation using a Hewlett-Packard G1005A automated protein sequencing system, calibrated with PTH-amino acid standards prior to each sequencing run.

The N-terminal amino acid sequence of HPLC-purified recombinant human progastrin determined by Edman analysis was SQQPDAPL, which corresponded precisely to residues 6-13 of human progastrin. We conclude that the HPLC-purified recombinant human progastrin consists of residues 6-80 inclusive of human progastrin. Since the N-terminal sequence of human progastrin is SWKPRSQQPDAPL, it appears that thrombin has cleaved the peptide bond between the arginine residue at position 5 and the serine residue at position 6. This cleavage is consistent with the

preferred recognition sequence P4-P3-Pro-Arg/Lys.P1'-P2', where P3 and P4 are hydrophobic amino acids, and P1' and P2' are non-acidic amino acids (Chang, 1985).

Example 3 Binding of Progastrin to CCK Receptors

Binding of recombinant human progastrin to either the human CCK-A or human gastrin/CCK-B receptor was investigated by competition for the binding of ^{125}I-CCK$_8$ to transiently transfected COS cells.

COS cells were transiently transfected by the DEAE-dextran method as described previously (Mantamadiotis and Baldwin, 1994). One day before transfection, 5 x 10^5 COS cells were seeded in 10 cm plates such that on the day of transfection the cells were 60% confluent. On the day of transfection, the medium was aspirated and the cells were incubated at 37°C for 3 hours in Dulbecco's modified Eagle's medium (DME) containing 1 µg/ml pRFNeo plasmid DNA encoding either the human CCK-A or the human CCK-B receptor, 400 µg/ml DEAE-dextran, and 100 µM chloroquine. After incubation, the solution was aspirated and the cells were treated with 10% dimethyl sulphoxide for 1 min. The transfected cells were then grown in DME with 10% FCS overnight. On the next day, the transfected cells were replated onto a 24 well dish (20,000/well) and grown for a further 48 hours prior to the receptor binding assay.

Binding was measured by competition for binding of ^{125}I-CCK$_8$ labelled by the Bolton and Hunter method, as described by Kopin *et al.* (1992). Transfected COS cells were grown to 80-90% confluence as described above, washed in PBS, and then incubated for 80 min at 37°C in Hank's balanced salt solution containing ^{125}I-CCK$_8$ (10,000 cpm, 2.9 fmol, Amersham, Bucks., UK), 16 µM phenyl methyl sulphonyl fluoride and 0.1% bovine serum albumin. Cells were then washed twice with PBS and lysed with 200 µl 1 M NaOH. Lysates were counted in a γ-counter (LKB-Wallac, Turku, Finland) at 77% efficiency. Initial estimates of IC_{50} values, and of the levels of ^{125}I-CCK$_8$ bound in the

absence of competitor, were obtained with the program SigmeStat (Jandel Scientific, San Rafael, CA) by non-linear regression to the equation

$$y=a/(1+x/b),$$

where

y is the amount of iodinated gastrin, expressed as a percentage of the value,

a observed in the absence of unlabelled peptide,

x is the concentration of unlabelled peptide, and

b is the IC_{50} value.

Binding of progastrin to the gastrin/CCK-C receptor was measured by covalent cross-linking of ^{125}I-[Nle_{15}]-$gastrin_{2,17}$ with disuccinimidylsuberate (Baldwin *et al*, 1986) in the presence of increasing concentrations of progastrin. Reaction products were separated by SDS-polyacrylamide gel electrophoresis and the radioactivity associated with the fusion proteins was detected and quantified with a BAS3000 phosphorimager (Fuji, Japan).

CCK_8 and $gastrin_{17}$ were used as positive controls for the CCK-A and gastrin/CCK-B receptors, respectively. Dose-dependent displacement of ^{125}I-CCK_8 from specific binding sites on COS cells transfected with plasmids encoding either the CCK-A receptor or the gastrin/CCK-B receptor was observed in the presence of unlabeled CCK_8 or $gastrin_{17}$, respectively. The results are illustrated in Figure 4. Recombinant human progastrin had no effect on the binding of ^{125}I-CCK_8 to either CCK-A receptor or CCK-B receptor, even at concentrations as high as 100 nM.

In contrast, recombinant human progastrin was able to bind to the gastrin/CCK-C receptor, as shown in Figure 5. The fact that the affinities of progastrin and glycine-extended $gastrin_{17}$ for the gastrin/CCK-C receptor were very similar suggests that residues 6-54 and 73-80 do not contribute significantly to receptor binding.

Example 4 Proliferation Studies on YAMC Cells

A colorimetric assay (Mosmann, 1983) was used to measure cell proliferation. Briefly, YAMC cells were seeded in a 96 well plate at a density of 2000 cells/well in RPMI medium containing 10% FCS. The following day, cells were synchronized in G_0 by incubation for 24 hours in medium lacking FCS but containing 10 µM thioglycerol, 0.025 units/ml insulin and 1 µg/ml hydrocortisone. The medium was then replaced with fresh medium containing 1% fetal calf serum and the peptide(s) under investigation, and incubation was continued for 3 days. 10 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St.Louis, MO) was added per well, and the plate was incubated for 4 hrs at 37°C before the medium was discarded. 200 µl 0.04M HCl in isopropanol was added to lyse the cells, and the absorbance at 560 nm was read on a BioRad Model 550 Microplate reader (BioRad, Hercules, CA).

The results are shown in Figure 6. Recombinant human progastrin and glycine-extended $gastrin_{17}$ both stimulated YAMC cell proliferation in a dose-dependent manner, with a maximal stimulatory effect seen at 0.3 nM. Concentrations of either peptide higher than 1 nM were inhibitory.

Example 5 Proliferation Studies on Carcinoma Cell Line

The proliferative effects of progastrin were tested using the tumorigenic human colorectal carcinoma cell lines LIM1215 and LIM1899. The methods were as described in Example 4 above, except that incubation was for 5 days, and the medium was RPMI containing 10% non-born calf serum. The results are shown in Figure 7.

In LIM 1215 cells progastrin at concentrations between 10 fM and 1 pM significantly stimulated proliferation. No significant stimulation of proliferation was observed with higher concentrations of progastrin, or with $gastrin_{17}$gly at any concentration. In contrast progastrin at concentrations between 10 fM and 1 nM had no

effect on the proliferation of LIM 1899 cells.

Although in a preliminary experiment progastrin$_{6-80}$ had a similar proliferative effect on LIM1839 human gastric carcinoma cells to that observed with gastrin$_{17}$gly, this effect was subsequently found not to be reproducible.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

References cited herein are listed on the following pages, and are incorporated herein by this reference.

REFERENCES

Baldwin, G.S.
Comp. Biochem. Physiol., 1993 104B 55-61.

Baldwin, G.S.
Proc. Natl. Acad. Sci. USA., 1994 91 7593-7597.

Baldwin, G.S.
F.E.B.S. Letters., 1995 359 97-100.

Baldwin, G.S., Chandler, R., Scanlon, D.B. and Weinstock, J.
J. Biol. Chem., 1986 261 12252-12257.

Baldwin, G.S. and Zhang, Q.-X.
Cancer Res., 1992 52 2261-2267.

Boel, E., Vuust, J., Norris, F., Norris, K., Wind, A., Rehfeld, J.F. and Marcker, K.A.
Proc. Natl. Acad. Sci. USA., 1983 80 2866-2869.

Chang J-Y.
Eur. J. Biochem., 1985 151 217.

Ciccotosto, G.D., McLeish, A., Hardy, K.J. and Shulkes, A.
Gastroenterology, 1995 109 1142-1153.

Dockray, G.J., Varro, A. and Dimaline, R.
Physiol. Rev., 1996 76 767-798

Finley, G.G., Koski, R.A., Melhem, M.F., Pipas, J.M. and Meisler, A.I.
Cancer Res., 1993 53 2919-2926.

Frangioni, J.V. and Neel, B.G.
Analyt. Biochem., 1993 210 179-187.

Frucht, H., Gazdar, A.F., Park, J.-A., Oie, H. and Jensen, R.T.
Cancer Res., 1992 52 1114-1122.

Gardner, J.D., Knight, M., Sutliff, V.E. and Jensen, R.T.
Am. J. Physiol., 1985 248 G98-G102.

Hollande F., Imdahl A., Mantamadiotis T., Ciccotosto G.D., Shulkes A. and Baldwin G.S.
Gastroenterology, 1997 113 1576-1588.

Hoosein, N.M., Kiener, P.A., Curry, R.C., Rovati, L.C., McGilbra, D.K. and Brattain, M.G.
Cancer Res., 1988 48 7179-7183.

Imdahl, A., Mantamadiotis, T., Eggstein, S., Farthmann, E.H. and Baldwin, G.S.
Cancer Res. Clin. Oncol., 1995 121 661-666.

Kochman, M.L., DelValle, J., Dickinson, C.J. and Boland, C.R.
Biochem. Biophys. Res. Commun., 1992 189 1165-1169.

Kopin, A.S., Lee, Y.-M., McBride, E.W., Miller, L.J., Lu, M., Lin, H.Y., Kolakowski, L.F. and Beinborn, M.
Proc. Natl. Acad. Sci. USA., 1992 89 3605-3609.

Magous, R. and Bali, J.-P.
Eur. J. Pharmacol., 1982 82 47-54.

Mantamadiotis, T. and Baldwin, G.S.
Biochem. Biophys. Res. Commun., 1994 201 1382-1389.

Matsumoto, M., Park, J., Sugano, K. and Yamada, T.
Am. J. Physiol., 1987 252 G315-G319.

Matsushima, Y., Kinoshita, Y., Nakata, H., Inomoto-Naribayashi, Y., Asahara, M., Kawanami C., *et al.* Jpn. J. Cancer Res., 1994 85 819-824.

Mosmann, T. J. Immunol. Methods., 1983 65 55-63.

Nemeth, J., Taylor, B., Pauwels, S., Varro, A. and Dockray, G.J. Gut, 1993 34 90-95.

Rehfeld, J.F. and Johnsen, A.H. Eur. J. Biochem., 1994 223 765-773

Seet, L., Fabri, L., Nice, E.C. and Baldwin, G.S. Biomed. Chromatog., 1987 2 159-163.

Seva, C., Dickinson, C.J. and Yamada, T. Science, 1994 265 410-412.

Silvente-Poirot, S., Dufresne, M., Vaysse, N. and Fourmy, D. Eur. J. Biochem., 1993 215 513-529.

Singh, P., Owlia, A., Espeijo, R and Dai, B. J. Biol. Chem., 1995 270 8429-8438.

Singh P., Owlia A., Varro A., Dai B., Rajaraman S. and Wood T. Cancer Res., 1996 56 4111-4115

Smith, D.B. and Johnson, K.S. Gene., 1988 67 31-40.

Upp, J.R., Singh, P., Townsend, C.M. and Thompson, J.C. Cancer Res., 1989 49 488-492.

Van Solinge, W.W., Nielsen, F.C., Friis-Hansen, L., Falkmer, U.G. and Rehfeld, J.F.
Gastroenterology, 1993 104 1099-1107.

Wang T.C., Koh T.J., Varro A., Cahill R.J., Dangler C.A., Fox J.G. and Dockray G.J.
J. Clin. Invest., 1996 98 1918-1929.

Weinstock, J. and Baldwin, G.S.
Cancer Res., 1988 48 932-937.

CLAIMS

1. Purified recombinant human progastrin, or a biologically-active non-amidated fragment thereof.

2. Purified recombinant human progastrin according to Claim 1, comprising amino acid residues 1-80 of the complete human progastrin sequence, or a biologically-active non-amidated fragment thereof.

3. Purified recombinant human progastrin according to Claim 1, comprising amino acid residues 6-80 of the complete human progastrin sequence, or a biologically-active non-amidated fragment thereof.

4. A method of preparing a recombinant human progastrin according to any one of Claims 1 to 3, comprising the steps of:

a) expressing human progastrin in a host cell as a fusion protein with glutathione-S-transferase;

b) purifying the fusion protein by affinity chromatography on glutathione-agarose beads;

c) cleaving the recombinant progastrin from the fusion protein by incubation with thrombin; and optionally

d) purifying progastrin by reverse-phase high performance liquid chromatography.

5. A method according to Claim 4, in which the host cell is *Escherichia coli*.

6. An antibody directed against recombinant human progastrin according to any one of Claims 1 to 3.

7. An antibody according to Claim 6 directed against human progastrin$_{6-80}$.

8. An antibody according to Claim 6 or Claim 7, or a biologically-active fragment or derivative thereof, which inhibits binding of progastrin to cell surface receptors so as to inhibit progastrin-induced cell proliferation.

9. An antibody according to any one of Claims 6 to 8 which is a polyclonal antibody.

10. An antibody according to any one of Claims 6 to 8 which is a monoclonal antibody.

11. An antibody according to any one of Claims 6 to 10, labelled with a detectable marker.

12. An antibody according to Claim 11, in which the marker is a radioactive, fluorescent or chemiluminescent label.

13. A composition comprising a recombinant human progastrin according to any one of Claims 1 to 3, together with a pharmaceutically-acceptable carrier.

14. A composition comprising an antibody according to any one of Claims 6 to 12, together with a pharmaceutically-acceptable carrier.

15. A method of diagnosis of a condition associated with hyperprogastrinanaemia, comprising the step of detecting progastrin in a biological sample.

16. A method of diagnosis of a condition associated with hyperactivity of autocrine stimulation, proliferation or activity of cells of the gastrointestinal mucosa, comprising the step of detection of elevated levels of progastrin in a biological sample.

17. A method according to Claim 15 or Claim 16 in which the condition is colorectal carcinoma, duodenal ulcer or gastrinoma.

18. A method according to any one of Claims 15 to 17, in which progastrin is detected using immunoassay.

19. A method according to Claim 18, in which the immunoassay utilises an antibody which binds to a region of the progastrin molecule within the sequence comprising amino acid residues 6-80 of the human progastrin sequence.

20. A diagnostic kit comprising an antibody according to any one of Claims 6 to 10, in which the antibody is labelled with a detectable marker.

21. A diagnostic kit according to Claim 18, in which the detectable marker is a radioactive, fluorescent or chemiluminescent label.

22. A method of treatment of a condition associated with hyperactivity of autocrine stimulation of proliferation of cells of the gastrointestinal mucosa,

comprising the step of administering an effective amount of an antagonist of binding of progastrin to gastrin/CCK-C receptors or other progastrin receptors to a mammal in need of such treatment.

23. A method according to Claim 22, in which the condition is associated with hyperprogastrinanaemia.

24. A method according to Claim 22 or Claim 23, in which the condition is selected from the group consisting of colorectal carcinoma, duodenal ulcer, gastrinoma, and side-effects of treatment with ATP blockers.

25. A method according to any one of Claims 22 to 24, in which the antagonist is an antibody directed against progastrin.

26. A method according to Claim 25 in which the antagonist is a monoclonal antibody, or a biologically-active fragment or recombinant derivative thereof.

27. A method of treatment of a degenerative condition of the gastrointestinal mucosa, comprising the step of administering an effective amount of recombinant human progastrin according to any one of Claims 1 to 3, to a mammal in need of such treatment.

28. A method according to Claim 27, in which the condition is ulcerative colitis or inflammatory bowel disease.

29. Use of an antagonist of binding of progastrin to gastrin/CCK receptor or other progastrin receptors in the manufacture of a medicament for the treatment of a condition associated with hyperactivity of autocrine stimulation of proliferation of cells of the gastrinintestinal mucosa.

30. Use according to Claim 29, in which the antagonist is an antibody directed against progastrin.

31. Use according to Claim 29 or Claim 30, in which the antagonist is a monoclonal antibody or a biologically-active fragment or recombinant derivative thereof.

32. Use of progastrin according to any one of Claims 1 to 3 in the manufacture of a medicament for the treatment of a degenerative condition of the gastrointestinal mucosa.

33. Use according to Claim 32, in which the condition is ulcerative colitis or inflammatory bowel disease.

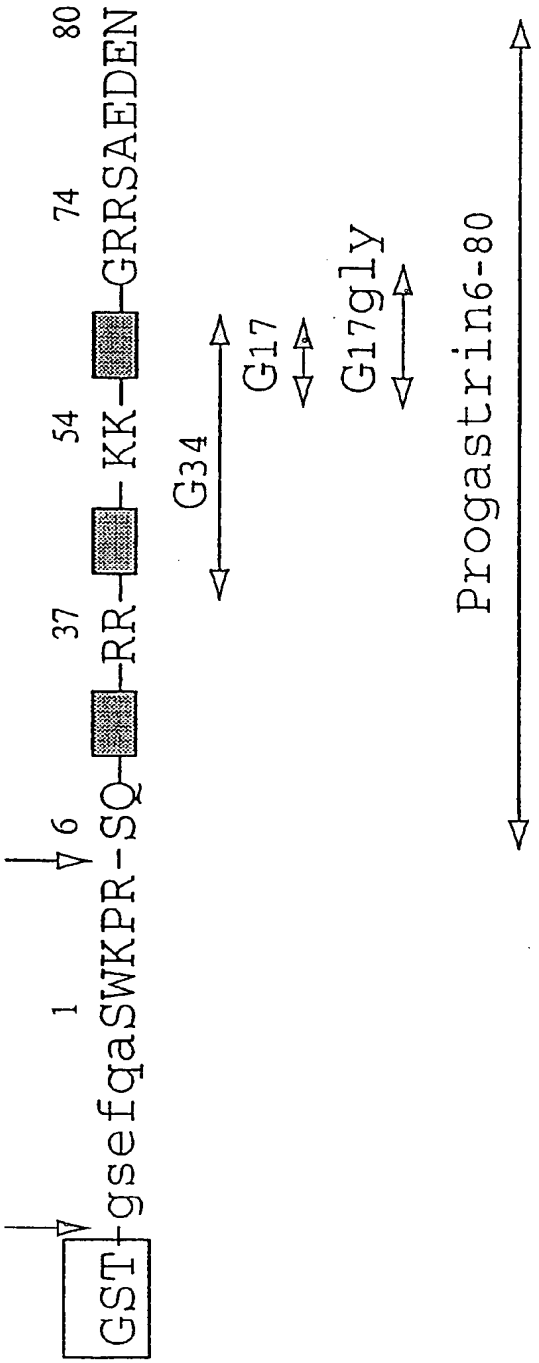
GST
gsefqaSWKPR-SQ-RR-KK-GRRSAEDEN
1
6
37
54
74
80
G34
G17
G17gly
Progastrin6-80

FIGURE 1

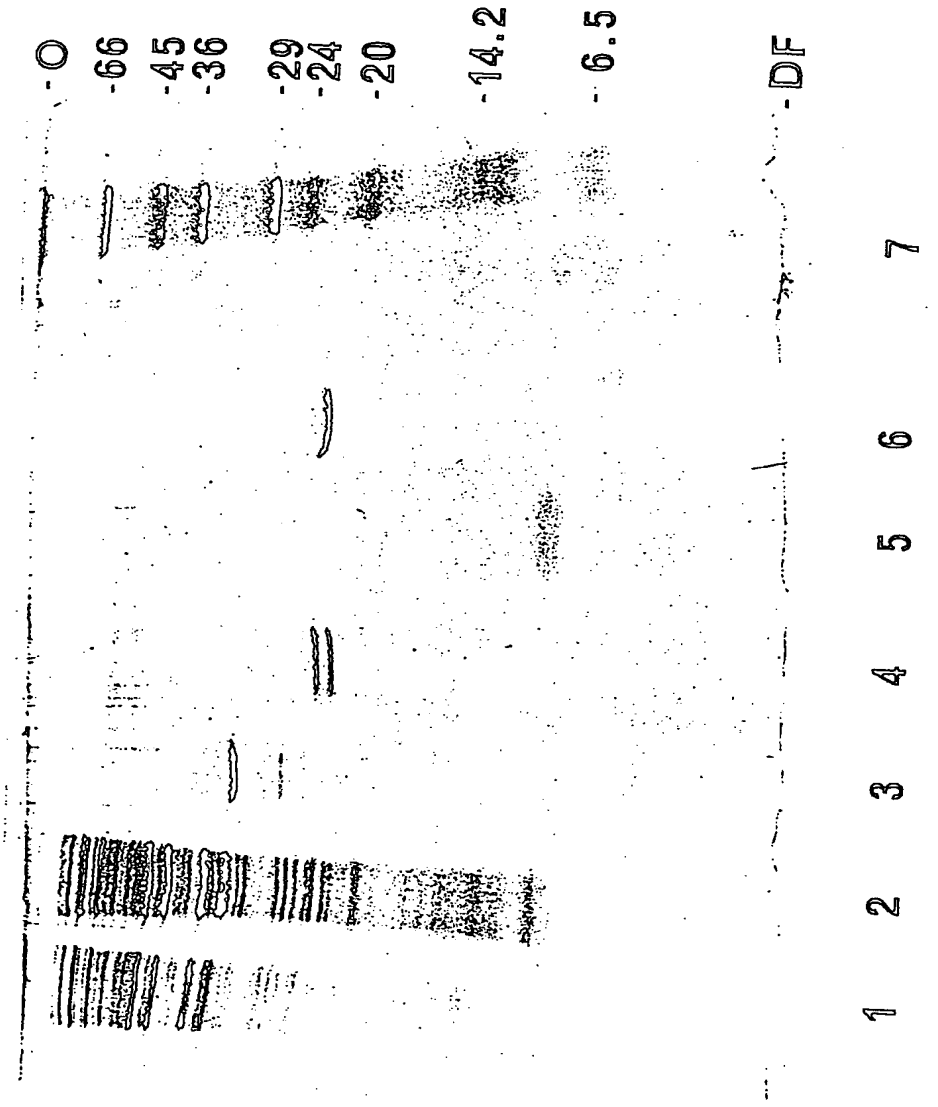

FIGURE 2

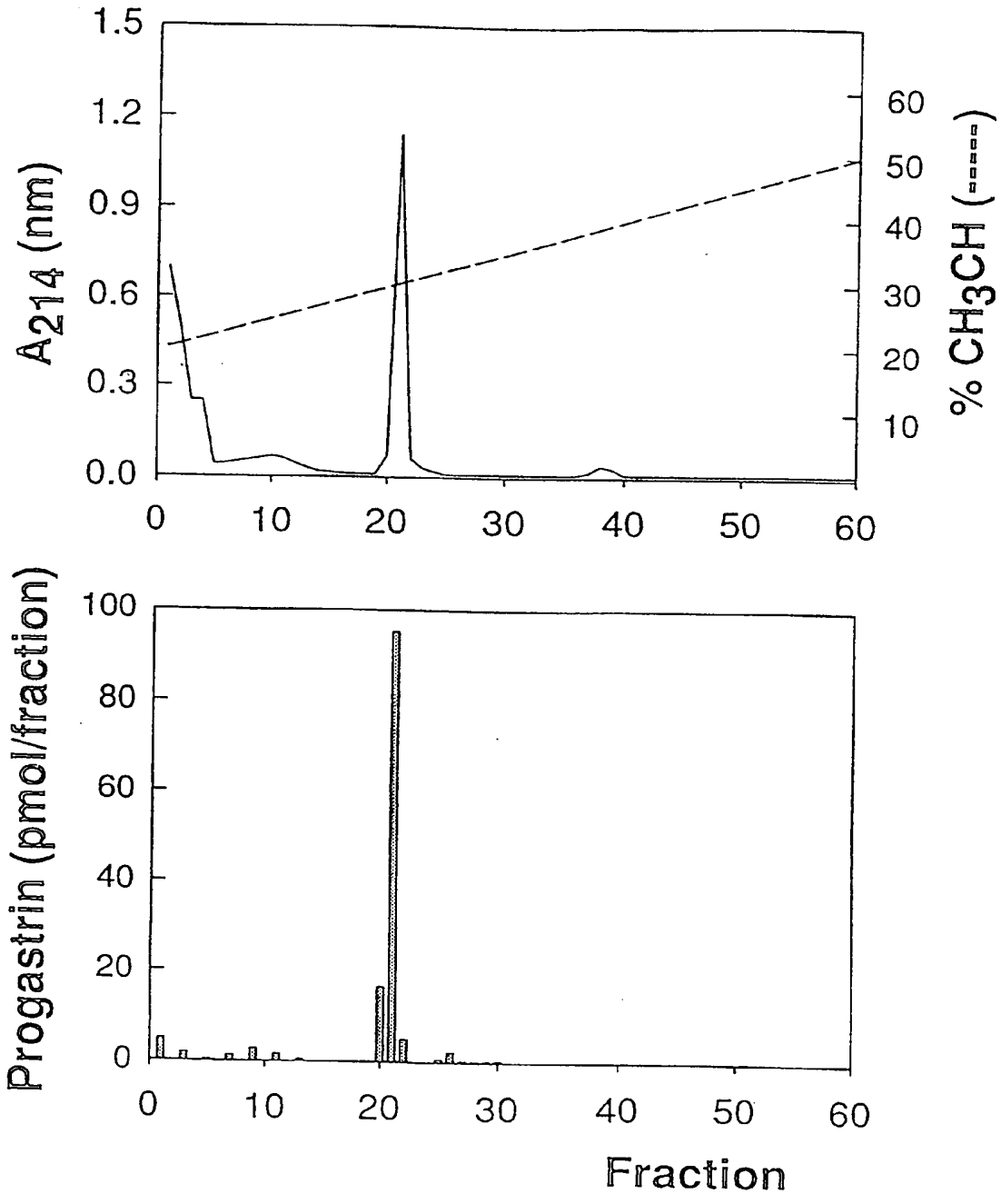
1.5
1.2
0.9
0.6
0.3
0.0
A214 (nm)
60
50
40
30
20
10
% CH3CH (-----)
0
10
20
30
40
50
60
100
80
60
40
20
0
Progastrin (pmol/fraction)
Fraction

FIGURE 3

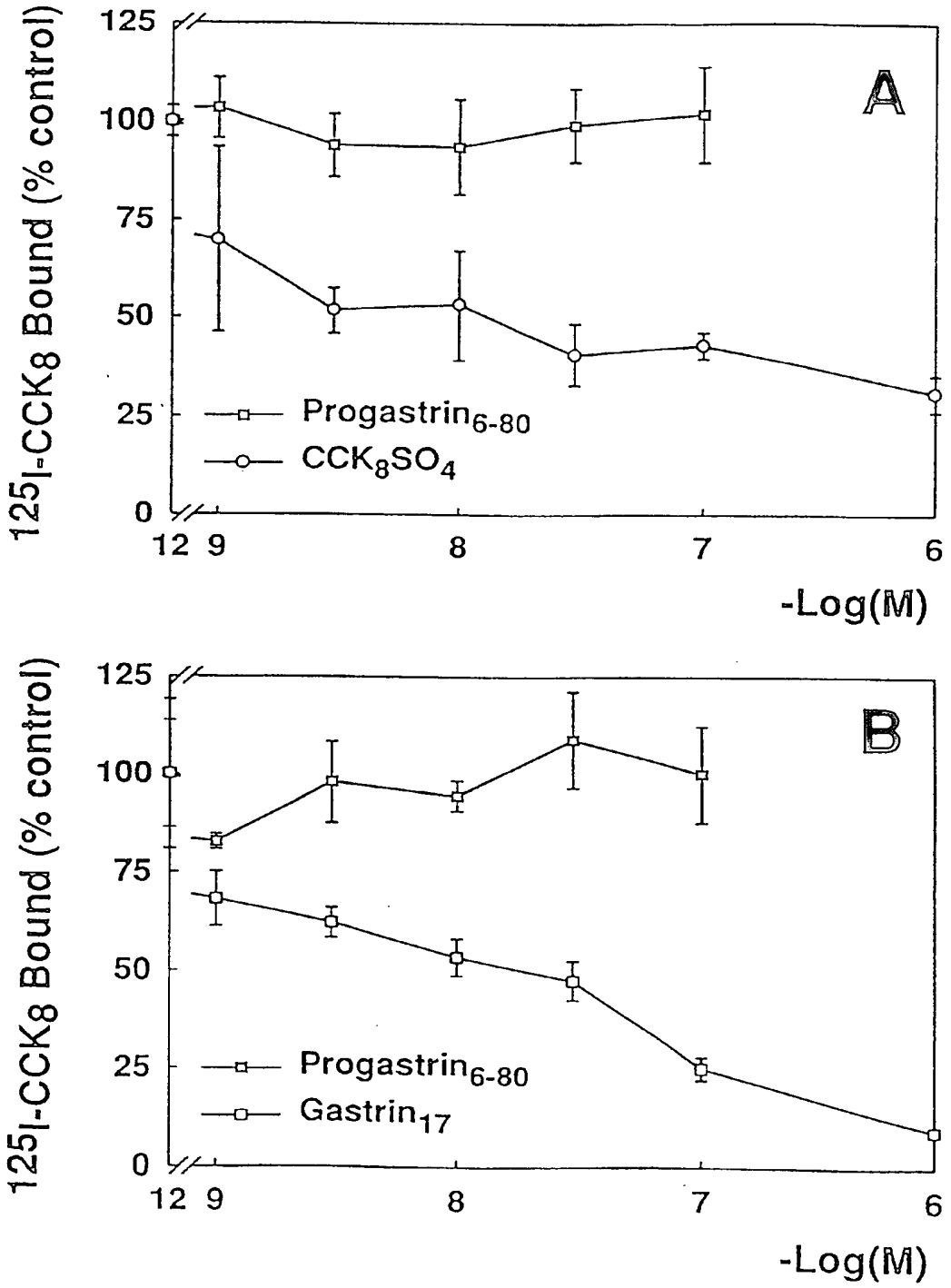
A
125
100
75
50
25
0
12 9 8 7 6
-Log(M)
^{125}I-CCK$_8$ Bound (% control)
Progastrin$_{6-80}$
CCK$_8$SO$_4$
B
125
100
75
50
25
0
12 9 8 7 6
-Log(M)
^{125}I-CCK$_8$ Bound (% control)
Progastrin$_{6-80}$
Gastrin$_{17}$

FIGURE 4

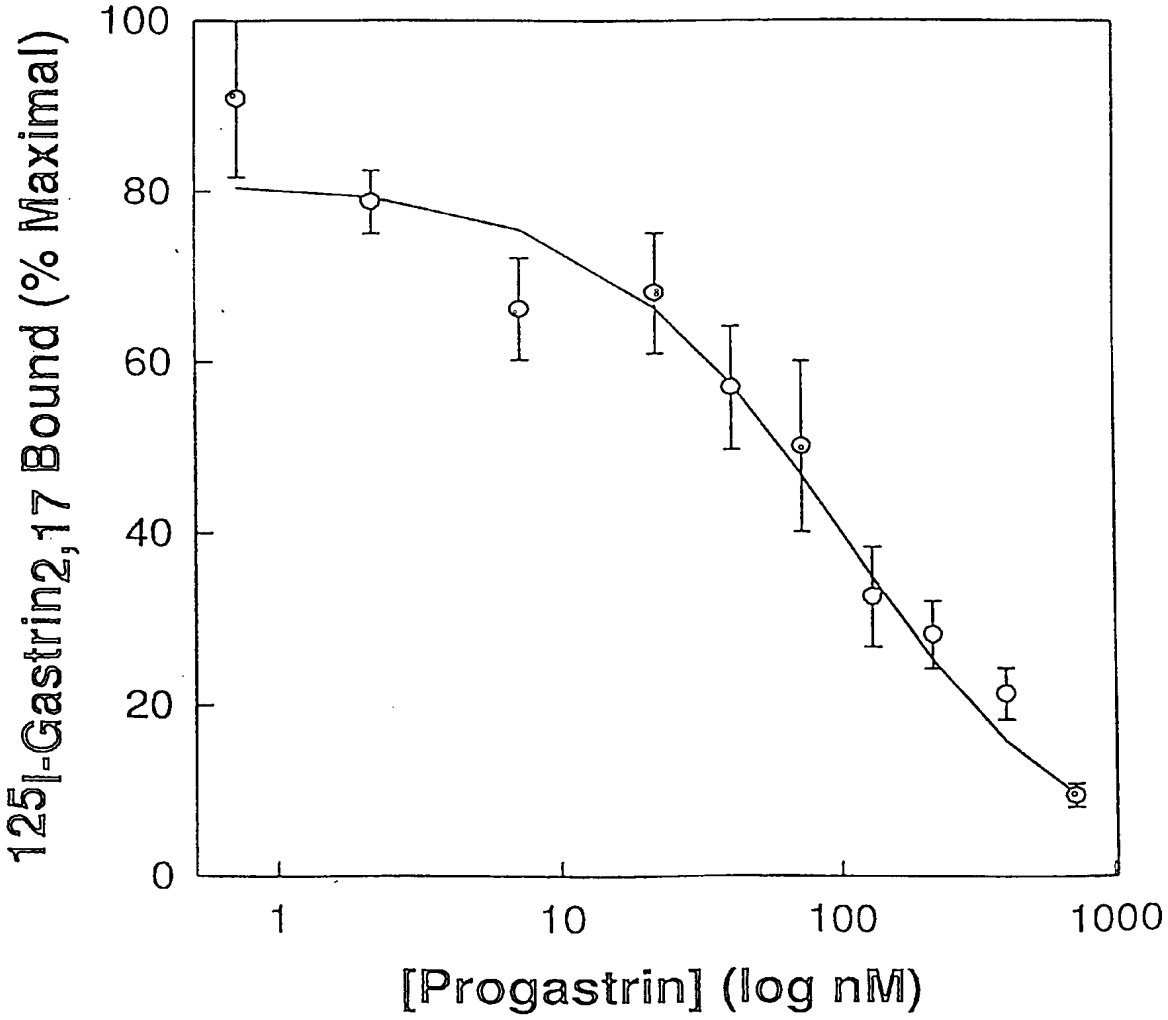
100
80
60
40
20
0
1
10
100
1000
^{125}I-Gastrin2,17 Bound (% Maximal)
[Progastrin] (log nM)

FIGURE 5

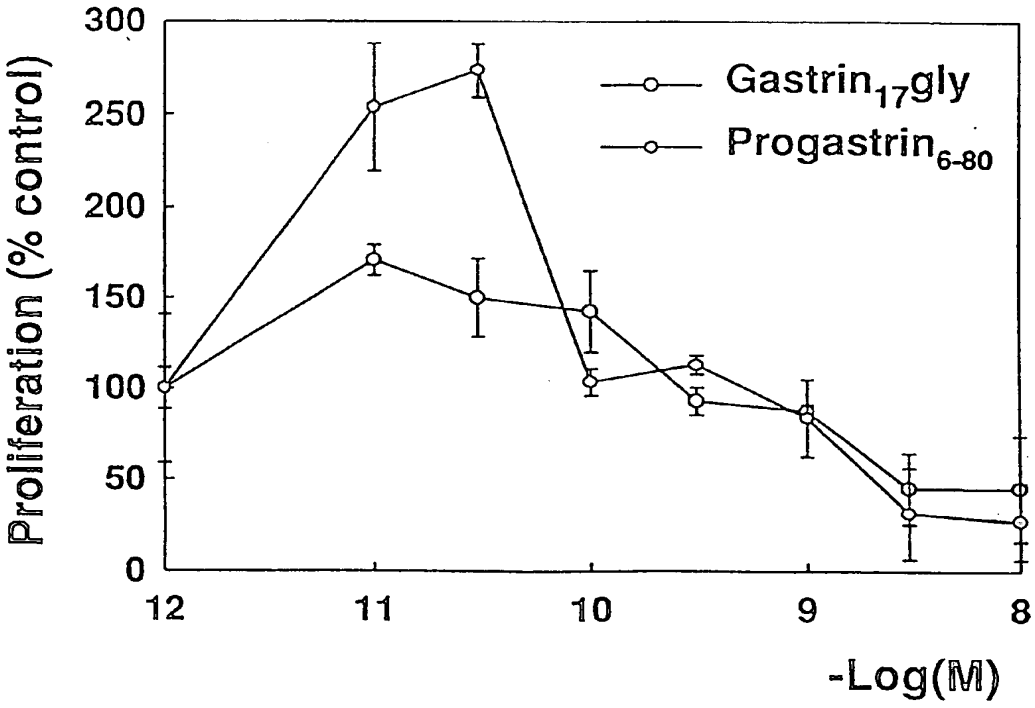
Proliferation (% control)
300
250
200
150
100
50
0
12
11
10
9
8
-Log(M)
Gastrin17gly
Progastrin6-80

FIGURE 6

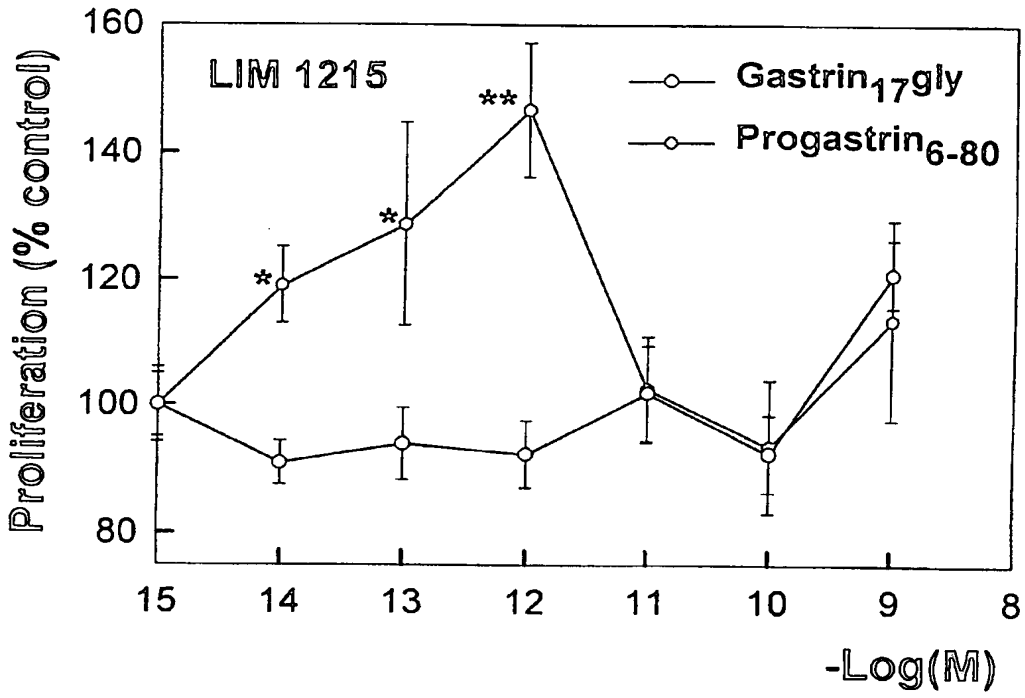
LIM 1215
Gastrin17gly
Progastrin6-80
Proliferation (% control)
160
140
120
100
80
15
14
13
12
11
10
9
8
-Log(M)
*
*
**

FIGURE 7a

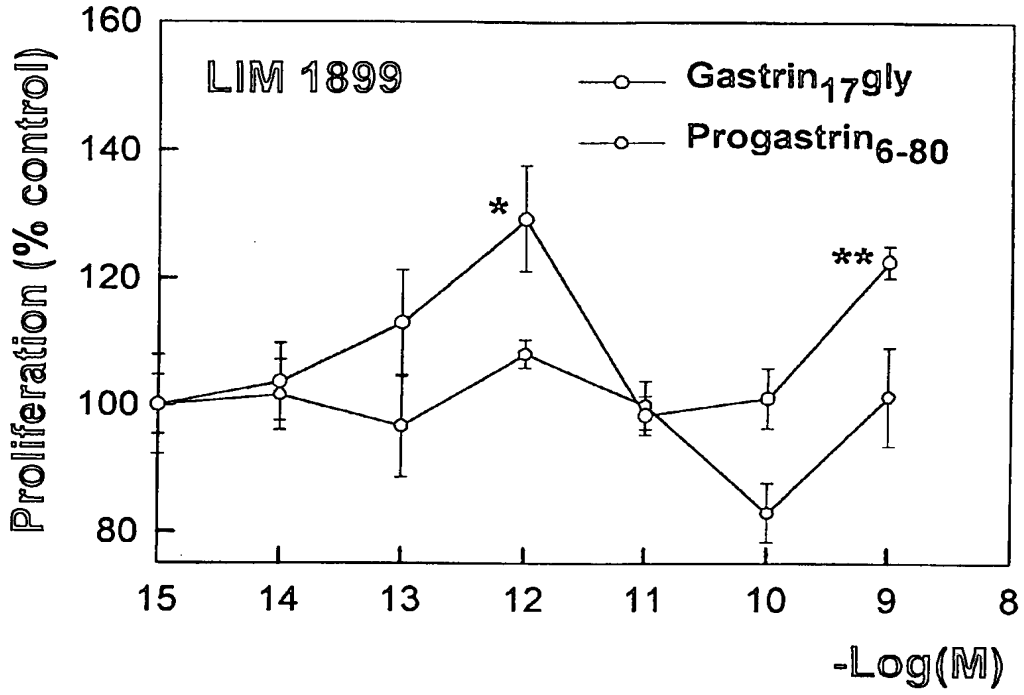
LIM 1899
Gastrin17gly
Progastrin6-80
Proliferation (% control)
160
140
120
100
80
15
14
13
12
11
10
9
8
-Log(M)
*
**

FIGURE 7b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 98/00851

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C07K 14/595, 16/26; A61K 38/22, 39/395; G01N 33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC as above

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN: Keywords Progastrin(s) and human(s)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Gastroenterology (1997) 113(5), pages 1576-1588, F. Hollande et al., "Glycine-Extended Gastrin Acts as as Autocrine Growth Factor in a Nontransformed Colon Cell Line".	1-32
X	The Journal of Clinical Investigation (1996) 98(8) pages 1918-1929, T. C. Wang et al., "Processing and Proliferative Effects of Human Progastrin in Transgenic Mice".	1-32
X	American Journal of Physiology (1991) 260(5) pages G783-G788, D. F. Daugherty et al., "Expression and Processing of Human Preprogastrin in Murine Medullary Thyroid Carcinoma Cells".	1-21,27-28

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 November 1998	30 NOV 1998
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer G. A. Jenkins GILLIAN JENKINS Telephone No.: (02) 6283 2252

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 98/00851

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Gastroenterology (1995) 109(4), pages 1142-1153, G. D. Ciccotosto et al., "Expression Processing and Secretion of Gastrin in Patients with Colorectal Carcinoma".	1-21
X	FEBS Letters(1994) 337(1), pages 27-32, N. Hayashi et al., "Production of Bioactive Gastrin from the Non-Endocrine Cell Lines CHO and COS-7".	1-21
X	European Journal of Biochemistry (1994) 223(3) pages 765-773, J. F. Rehfeld et al., "Identification of Gastrin Component I as Gastrin-71. The Largest Possible Bioactive Progastrin Product".	1-3,6,8-14,20-21